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Binding and degradation of fibrinogen by *Bacteroides fragilis* and characterization of a 54 kDa fibrinogen-binding protein

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Bacteroides fragilis is a bacterium that resides in the normal human gastro-intestinal tract; however, it is also the most commonly isolated Gram-negative obligate anaerobe from human clinical infections, such as intra-abdominal abscesses, and the most common cause of anaerobic bacteraemia. Abscess formation is important in bacterial containment, limiting dissemination of infection and bacteraemia. In this study, we investigated *B. fragilis* binding and degradation of human fibrinogen, the major structural component involved in fibrin abscess formation. We have shown that *B. fragilis* NCTC9343 binds human fibrinogen. A putative *Bacteroides fragilis* fibrinogen-binding protein, designated BF-FBP, identified in the genome sequence of NCTC9343, was cloned and expressed in *Escherichia coli*. The purified recombinant BF-FBP bound primarily to the human fibrinogen Bβ-chain. In addition, we have identified fibrinogenolytic activity in *B. fragilis* exponential phase culture supernatants, associated with fibrinogenolytic metalloproteases in NCTC9343 and 638R, and cysteine protease activity in YCH46. All nine clinical isolates of *B. fragilis* examined degraded human fibrinogen; with eight isolates, initial Aα-chain degradation was observed, with varying Bβ-chain and γ-chain degradation. With one blood culture isolate, Bβ-chain and γ-chain degradation occurred first, followed by subsequent Aα-chain degradation. Our data raise the possibility that the fibrinogen-binding protein of *B. fragilis*, along with a variety of fibrinogenolytic proteases, may be an important virulence factor that facilitates dissemination of infection via reduction or inhibition of abscess formation.

INTRODUCTION

Bacteroides fragilis is the Gram-negative obligate anaerobe of the normal human intestinal microbiota most frequently isolated from opportunistic infections (Patrick, 2002; Patrick & Duerden, 2006). These include peritonitis, soft tissue abscesses and bacteraemia, with an estimated mortality rate of 19 % (Patrick *et al.*, 1995; Redondo *et al.*, 1995). During peritonitis, intra-abdominal abscesses composed primarily of a thick fibrin wall are formed from the conversion of fibrinogen, a key structural component of the blood coagulation system, to fibrin. Human fibrinogen is a 340 kDa glycoprotein, composed of three pairs of non-identical polypeptide chains: the Aα-chain (610 amino acids, ~67 kDa), the Bβ-chain (461 amino acids, ~55 kDa) and the γ-chain (411 amino acids, ~48 kDa) (Henschen *et al.*, 1983), bound together by 29 disulphide bridges. A and B denote the N-terminal peptides that are cleaved by thrombin from the Aα and Bβ chains when fibrin is formed (Weisel *et al.*, 1985; Weisel, 2005). Bacteria, in the range of 10^7–10^9 ml^{-1}, in addition to white blood cells, tissue debris and inflammatory exudates, can be sequestered within an abscess (Tally & Ho, 1987). Inhibition of formation and abscess rupture are likely to be important for the dissemination of infection and bacteraemia. Pathogenic factors involved in *B. fragilis* dissemination from the initial site of infection have yet to be identified conclusively, but interference with blood clotting proteins may be particularly important.

Several bacterial cell-surface adhesin proteins capable of binding to human fibrinogen have been linked to virulence (Patti *et al.*, 1994). These include *Staphylococcus aureus*

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRR, leucine-rich repeat region; TVC, total viable count.

proteins FnbpA (fibronectin-binding protein A) (Wann *et al.*, 2000) and clumping factor A (ClfA), which bind to the C terminus of the fibrinogen γ-chain. *Tannerella forsythia*, a Gram-negative bacterium associated with advanced and recurrent human periodontitis, expresses an outer membrane surface protein, BspA, associated with fibrinogen binding (Sharma *et al.*, 1998; Inagaki *et al.*, 2006; Honma *et al.*, 2001). *B. fragilis* has been shown to bind to the extracellular components, fibronectin, collagen, vitronectin (Nagy *et al.*, 1994; Szoke *et al.*, 1996) and laminin (Eiring *et al.*, 1995). ELISA analyses have shown that intact *B. fragilis* BE1 cells are capable of binding plasminogen in a dose-dependent manner and plasminogen outer membrane proteins have been described (Ferreira Ede *et al.*, 2009; Sijbrandi *et al.*, 2005).

In addition to fibrinogen-binding proteins, putative fibrinogenolytic virulence factors have also been identified in pathogenic Gram-positive bacteria (e.g. Matsuka *et al.*, 1999) and in Gram-negative bacteria including anaerobes [e.g. the Arg-gingipains (RgpA and RgpB) and the Lys-gingipain Kgp/PrtP of the periodontitis-associated bacterium *Porphyromonas gingivalis* (Barkocy-Gallagher *et al.*, 1999; Lantz *et al.*, 1991)]. The release of extracellular enzymes from *Bacteroides* spp. with the potential to degrade components of the host has been long recognized (reviewed by Patrick, 2002). Enzymic activity associated with copious amounts of single membrane-bound outer membrane vesicles produced by *B. fragilis* has also been described (Patrick *et al.*, 1996; Domingues *et al.*, 1997). Chen *et al.* (1995) have demonstrated the fibrinogenolytic activity of crude *B. fragilis* cellular extracts and described a putative 100 kDa monomeric serine-thiol-like fibrinogenolytic protease from *B. fragilis* YCH46, capable of hydrolysing the substrates azocasein, casein, gelatin, azocoll and fibrinogen, but unable to degrade BSA, ovalbumin, fibrin, fibronectin, immunoglobulins, transferrin, haemoglobin and collagen types I, III and IV. Whether this activity related to extracellular or intracellular enzymes was not resolved. The secreted 20 kDa zinc metalloprotease enterotoxin, which is associated with acute diarrhoeal disease in humans and animals (Myers *et al.*, 1984; Myers & Shoop, 1987; Border *et al.*, 1985), is also reported to have fibrinogenolytic activity. The role of *B. fragilis* enterotoxin is still unclear, however, as the enterotoxin gene has only been detected in 18 % of *B. fragilis* clinical isolates from Poland, the UK, Holland and France (Luczak *et al.*, 2001). There are reports, however, that the enterotoxin gene is present more frequently in bacteraemia isolates than in non-blood isolates (Claros *et al.*, 2006).

We now present evidence that *B. fragilis* is capable of binding human fibrinogen, an interaction which may be mediated via a novel fibrinogen-binding putative outer surface protein, *Bacteroides fragilis*-fibrinogen binding protein, which we designate BF-FBP. We also demonstrate exponential phase proteolysis of fibrinogen by *B. fragilis* extracellular proteases not related to the *B. fragilis* enterotoxin.

METHODS

Bacterial strains and culture conditions. Isolates studied included: *B. fragilis* National Collection of Type Cultures (NCTC) 9343, obtained from the NCTC, London, UK; *B. fragilis* 638R, a spontaneous rifampicin-resistant mutant of strain 638 (Privitera *et al.*, 1979), isolated from an abdominal abscess and kindly gifted by C. J. Smith, University of East Carolina, USA; *B. fragilis* YCH46, a bacteraemia isolate from Yamaguchi Prefecture, Japan, was kindly gifted by T. Kuwahara, University of Tokushima, Japan; and *B. fragilis* SP1, SP2, DK9, LS98, LS66 and LS27, clinical isolates recovered from patients in Northern Ireland. *Escherichia coli* DH5α (Invitrogen) was used for standard cloning protocols. *B. fragilis* was cultured in either supplemented brain heart infusion (BHI-S) or defined medium (DM) broth (Van Tassell & Wilkins, 1978). *B. fragilis* was cultured in a MACS MG-1000 anaerobic workstation (Don Whitley) at 37 °C in an atmosphere of 80 % nitrogen, 10 % carbon dioxide and 10 % hydrogen. *E. coli* was cultured using BHI and Luria–Bertani (LB) medium at 37 °C.

B. fragilis* fibrinogen-binding assay.** Fibrinogen binding by *B. fragilis* strain NCTC9343 was investigated by immunofluorescence microscopy using *B. fragilis* cells grown to late-exponential phase in BHI-S broth. Cells were washed in sterile PBS by centrifugation at 1610 ***g for 20 min (×3) at room temperature, resuspended to OD_{600} 0.3, applied to Teflon-coated multiwell slides (30 µl; ICN Biomedicals), air-dried and fixed in 100 % methanol at −20 °C for 20 min. Duplicate slides were then incubated for 2 and 16 h at 37 °C with the following concentrations of human fibrinogen in ultrapure water: 30 µl of 25, 50, 75, 100, 500, and 1000 µg ml^{-1}. Slides were then washed in sterile PBS for 20 min. For blocking experiments, washed bacteria were pre-incubated with 100 µg human fibrinogen ml^{-1} and 1 mg human fibrinogen ml^{-1} at 37 °C for 2 and 48 h, respectively, washed by centrifugation at 1610 ***g*** for 10 min (three times) in sterile PBS and resuspended (OD_{600} 0.3), and 30 µl was fixed to multiwell slides as described above. Slides prepared as described above were then incubated with goat anti-human fibrinogen polyclonal antiserum (30 µl of 1 : 3000 dilution, Sigma) at 37 °C for 1 h, washed for 20 min in PBS and then incubated for 1 h at 37 °C with FITC-conjugated rabbit anti-goat IgG antibody whole molecule (30 µl of 1 : 100 dilution, Sigma) containing 0.2 % (v/v) Evans Blue counter stain. Slides were washed and mounted in a glycerol–PBS anti-bleaching mounting fluid (Citifluor, Agar Scientific) and examined at ×1000 magnification (Leitz Ortholux fluorescence microscope). Images were captured using a Nikon DMX 1200 digital camera and Lucia G/F software.

Recombinant BF1705 (rBF-FB) protein expression and purification. The mature BF1705 coding sequence (with signal sequence and prokaryotic membrane lipoprotein lipid attachment sites truncated) was amplified from *B. fragilis* NCTC9343 genomic DNA using the forward primer 5′-CAGGATCATATGAAAGACTCTCC-AAACGAATTAA-3′ and the reverse primer 5′-CAGGATGGATC-CCAGACCTCAAAGAGCAACG-3′, and ligated into the *Nde*I and *Bam*HI sites of the pET15b N-terminal His-tag expression vector (Novagen). *E. coli* BL21(DE3)-Gold (Stratagene) competent cells were transformed with the expression vector. The pET15b-BF1705 clone was confirmed by DNA sequencing. A single transformant was cultured in 10 ml LB broth containing 100 µg ampicillin ml^{-1}, inoculated into 1 l and incubated at 16 °C for 16 h at 200 r.p.m. in an orbital incubator (Gallenkamp). Recombinant protein was purified by affinity column chromatography using nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen) according to the manufacturer's instructions, flash-frozen and stored at −80 °C.

Protein dot-blot assays and far-Western analysis. rBF-FB purified protein was dialysed against sodium bicarbonate buffer

(50 mM $NaHCO_3$, pH 8.5) and labelled with *N*-hydroxysuccinimide (NHS)-biotin (Sigma). For dot-blot overlay assays, twofold serial dilutions consisting of 4.0 μg, 2.0 μg, 1.0 μg, 0.5 μg, 0.25 μg, 0.125 μg, 62.5 ng, 31.2 ng and 0 (negative control) plasminogen-free human fibrinogen, each mixed with 4 μg BSA, were blotted onto nitrocellulose membranes (Hybond-C Extra, 45 μm pore-size, Amersham Biosciences). To demonstrate binding of rBF-FB to fibrinogen after SDS-PAGE and blotting (far-Western analysis), plasminogen-free human fibrinogen was electrophoresed on a 10 % (w/v) SDS-PAGE gel and transferred to a nitrocellulose membrane, washed and blocked as described above. Both dot and far-Western blots were incubated with gentle rocking in 10 μg ml^{-1} solutions of biotinylated BF1705 recombinant protein, washed and then incubated for 30 min at room temperature with 1 : 500 streptavidin–alkaline phosphatase conjugate [1 : 500 dilution in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1 % (v/v) Tween-20 (Sigma)]. After washing, blots were treated with *Fast* BCIP/NBT substrate (Sigma) according to the manufacturer's instructions. The experiment was repeated on two separate occasions.

Fibrinogen degradation by cell-free supernatants and outer membrane protein extracts. Late-exponential phase and stationary phase *B. fragilis* strains NCTC9343, 638R and YCH46 in BHI-S or DM broth were harvested at 4 °C by centrifugation at 1610 ***g*** for 25 min. After filter-sterilization, supernatants were concentrated (exponential phase supernatants, 22-fold; stationary phase, 15-fold) at 4 °C using 10 kDa molecular weight cut-off (MWCO) centrifugal filters (Amicon Centriplus, Millipore). BHI-S broth and DM broth were prepared as described above.

Outer membrane protein extracts were prepared using Sarkosyl as previously described (Patrick & Lutton, 1990). This was repeated to ensure that all potential contaminating inner membrane proteins were removed by solubilization. The Sarkosyl-insoluble outer membrane pellet was washed three times in quarter-strength Ringer's solution containing L-cysteine (0.5 mg ml^{-1}) and suspended in outer membrane buffer (50 mM Tris/HCl, pH 7.5).

Plasminogen-free human fibrinogen (Sigma) was dissolved in 350 μl concentrated *B. fragilis* supernatant or outer membrane protein extracts (from ~ 4.0×10^9 c.f.u.) to a concentration of 100 μg ml^{-1}. The samples and the negative controls (fibrinogen at 100 μg ml^{-1} in concentrated BHI-S broth and outer membrane buffer) were incubated anaerobically for 48 h at 37 °C. Aliquots were removed after 0, 24 and 48 h and analysed by SDS-PAGE as described below. Experiments were repeated at least twice.

SDS-PAGE and immunoblot analysis of *in situ* fibrinogen degradation. Pre-reduced BHI-S broth containing 100 μg ml^{-1} plasminogen-free human fibrinogen (Sigma) was inoculated with *B. fragilis* strains NCTC9343, 638R, YCH46, DK9, LS66, LS27, SP1, SP2 and LS98. Post-inoculation samples (0.5 ml) were removed at 0, 3, 6, 9, 12, 24, 27, 30, 33 and 48 h and centrifuged at 16 100 ***g*** for 5 min at 4 °C, and the supernatants were separated aseptically from the cell pellet. Replicate total viable counts (TVCs) were determined using the Miles–Misra drop count method. Sterile BHI-S broth containing 100 μg ml^{-1} plasminogen-free human fibrinogen was incubated and sampled in parallel. Supernatants were analysed by SDS-PAGE [10 % (w/v) Bistris SDS-polyacrylamide gels (NuPAGE, Invitrogen)] at a constant voltage of 200 V in MOPS SDS running buffer and stained with Coomassie Blue. Immunoblotting was carried out using nitrocellulose (Protran 0.45 μm pore-size, Schleicher & Schuell) at constant voltage (30 V) for 1 h at room temperature. Blots were blocked for 1 h at 37 °C in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5), containing 5 % (w/v) dried semi-skimmed milk (Marvel) and 0.05 % (v/v) Tween-20, washed at 37 °C in TBS containing 0.05 % (v/v) Tween-20 (TBST), incubated with a delipidized goat anti-human fibrinogen polyclonal antiserum (1/ : 10 000 dilution, Sigma) for 1 h at 37 °C, washed in TBS/Tween (TBST) and incubated for 1 h at 37 °C with an alkaline phosphatase-conjugated rabbit anti-goat IgG polyclonal antibody (1 : 20 000 dilution whole molecule, Sigma) and again washed. The detection substrate was Sigma *Fast* BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma). Experiments were repeated at least twice.

Densitometry analysis of fibrinogen degradation. The freely available ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij/index.html) was used to perform densitometry analysis of fibrinogen degradation. Briefly, images were opened in ImageJ and converted to greyscale (B-bit). Settings were as follows: background noise was corrected using a rolling ball radius of 50. Set scale/unit of length was set for pixels. Images were inverted (hence an increase in SDS-PAGE protein band intensity results in an increased measured grey value). Bands were then highlighted using the freehand selection tool in order to eliminate background or gel artefacts. Measurements of the area selected, the mean grey value and integrated density (area × mean grey area) in terms of pixels were calculated. The relative intensities of each fibrinogen chain after 0 and 24 h incubation detected by SDS-PAGE were calculated (mean grey value at t=0 h/mean grey value at t=24 h).

Fibrinogen zymography. Concentrated supernatants or outer membrane protein extracts as described above were prepared in a non-reducing treatment buffer and separated on 10 % (w/v) SDS-PAGE gels containing 0.1 % (w/v) plasminogen-free human fibrinogen (Sigma), for 1.5 h at 4 °C and a constant 125 V with a Tris-glycine running buffer [0.025 M Tris base, 0.192 M glycine, 0.1 % (w/v) SDS, pH 8.3] as previously described (Patrick *et al.*, 2009). Proteinases were renatured by washing twice in 2.5 % Triton X-100 (Sigma), followed by two washes in 50 mM Tris/HCl, pH 7.5, containing 2.5 % (v/v) Triton X-100 (Sigma) and one wash in 50 mM Tris/HCl, pH 7.5, for 15 min at room temperature. Gels were then incubated for 48 h at 37 °C in a serine protease activation buffer (50 mM Tris/HCl, pH 8.2, 150 mM NaCl, 5 mM $CaCl_2$), a metalloprotease activation buffer [50 mM Tris/HCl, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02 % (v/v) Brij-35 (polyoxyethyleneglycol dodecyl ether) (Sigma)] or a cysteine protease activation buffer [50 mM Tris/HCl, pH 6.4 or 7.5, 150 mM NaCl, 10 mM DTT (Sigma)/50 mM L-cysteine (Sigma)] (Barkocy-Gallagher *et al.*, 1999; Lantz *et al.*, 1991). Zones of lysis were detected by staining the gel for 2 h at room temperature [25 % (v/v) 2-propanol, 10 % (v/v) acetic acid, 64.9 % (v/v) ultrapure water and 0.1 % (w/v) Coomassie brilliant blue R-250 (BDH)] and destained for 16–24 h at room temperature [25 % (v/v) 2-propanol, 10 % (v/v) acetic acid, 65 % (v/v) H_2O] until optimum proteolytic band intensity, observed as clear bands against a dark-blue background, was achieved. For inhibitor studies, exponential phase supernatants from *B. fragilis* NCTC9343 and 638R grown in BHI-S broth (concentrated 33-fold) were incubated for 60 min at 37 °C in the presence or absence of 10 mM EDTA or 10 mM PMSF. The zymogram activation step was also carried out in the presence or absence of 10 mM EDTA. Experiments were repeated three times.

MS. His-tagged purified recombinant protein was loaded onto a Novex 10 % (w/v) Tris-glycine polyacrylamide gel (Invitrogen) and electrophoresed at a constant voltage of 200 V for 1.75 h at 4 °C in 1 × Tris-glycine SDS running buffer. Following electrophoresis, the gel was stained with Gelcode blue staining reagent (Pierce) without fixing according to the manufacturer's instructions. *B. fragilis* NCTC9343 and 638R were grown to late exponential phase in DM broth. Supernatant was concentrated 300-fold and separated by SDS-

PAGE as described above. Resolved protein bands were excised and prepared for MS by trypsin digestion according to the method of Aitken & Learmonth (2002).

Samples were subjected to MS analysis using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems). The peptide mass fingerprint spectra for each protein and subsequent protein identification were obtained using the MASCOT database (Matrix Science).

RESULTS

Analysis of human fibrinogen binding by *B. fragilis*

The interaction of *B. fragilis* with human fibrinogen was determined by incubating the bacterium with fibrinogen followed by labelling with an anti-human fibrinogen primary antibody and immunofluorescence microscopy. *B. fragilis* bound human fibrinogen, as indicated by anti-human fibrinogen antibody labelling of the bacterial cell surface (Fig. 1). Results were similar after incubation with human fibrinogen for either 2 or 24 h, suggesting that fibrinogen adhesion to the bacterial cell approaches saturation levels within 2 h of exposure. Non-specific binding was not evident, as immunofluorescent labelling of *B. fragilis* was not detected in the absence of fibrinogen (data not shown). This indicates that *B. fragilis* NCTC9343 is capable of interacting with human fibrinogen via one or more surface components. When *B. fragilis* NCTC9343 cells are pre-incubated with higher concentrations of human fibrinogen (1 mg ml^{-1}) aggregates are formed (Fig. 1c).

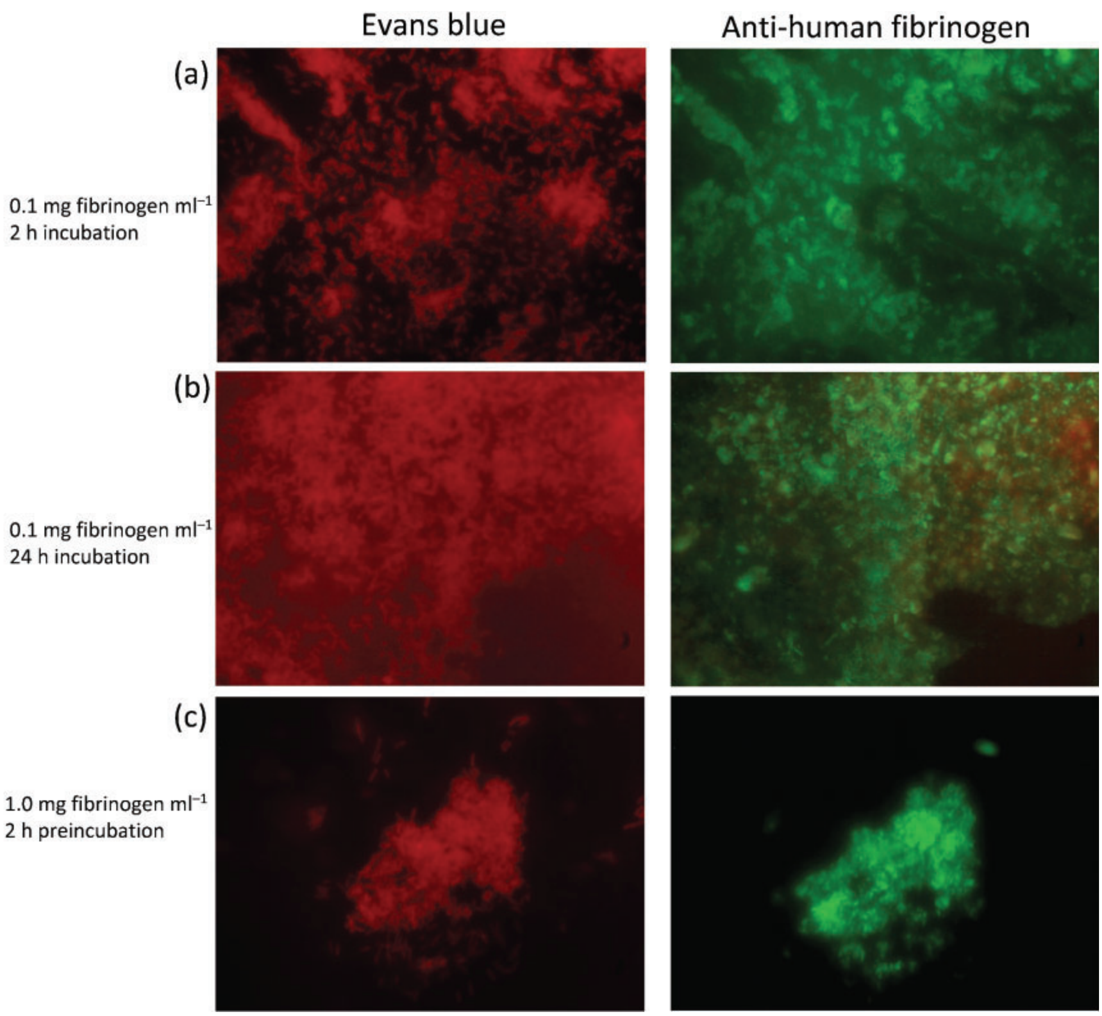

Fig. 1. Immunofluorescence micrographs of slide-fixed *B. fragilis* NCTC9343 incubated with (a) 0.1 mg human fibrinogen ml^{-1} for 2 h or (b) 0.1 mg human fibrinogen ml^{-1} for 24 h, and (c) *B. fragilis* NCTC9343 incubated with 1 mg human fibrinogen ml^{-1} for 2 h prior to slide fixation. Slides were reacted with FITC-conjugated anti-human fibrinogen polyclonal antiserum and counterstained with Evans Blue. Note the clumping phenotype when bacteria were pre-incubated with fibrinogen.

Identification, purification and functional characterization of a novel *B. fragilis* fibrinogen-binding protein

A putative 57 kDa outer membrane lipoprotein, encoded by BF1705, was identified in the genome sequence of strain NCTC9343 (Cerdeno-Tarraga *et al.*, 2005) as a potential fibrinogen-binding protein. Sequence analysis predicted that the protein contains a signal sequence and a membrane lipoprotein lipid attachment site, indicating that the molecule may be cell-surface-associated. The presence of two leucine-rich repeat regions (LRRs) in the sequence indicates probable protein–protein interactions (Kobe & Deisenhofer, 1994). In addition, the protein was predicted to be similar to the LRR-containing surface antigen BspA of *Tannerella forsythia*, which binds human fibrinogen (Sharma *et al.*, 1998). The mature 54 kDa *B. fragilis* protein, designated *Bacteroides fragilis*-fibrinogen binding protein (BF-FB), was purified to greater than 95 % purity (Fig. 2a); identity was confirmed by trypsin digestion and MS analysis. Binding of the recombinant BF-FB (rBF-FB) to human fibrinogen was examined by protein dot-blotting/overlay. The protein bound human fibrinogen in a dose-dependent manner, but failed to bind BSA (Fig. 2b). Binding of biotinylated rBF-FB to human fibrinogen after SDS-PAGE and blotting (far-Western analysis) revealed that the *B. fragilis* protein interacted strongly with the Bβ-chain of fibrinogen and weakly with the Aα- and γ-chains (Fig. 2c).

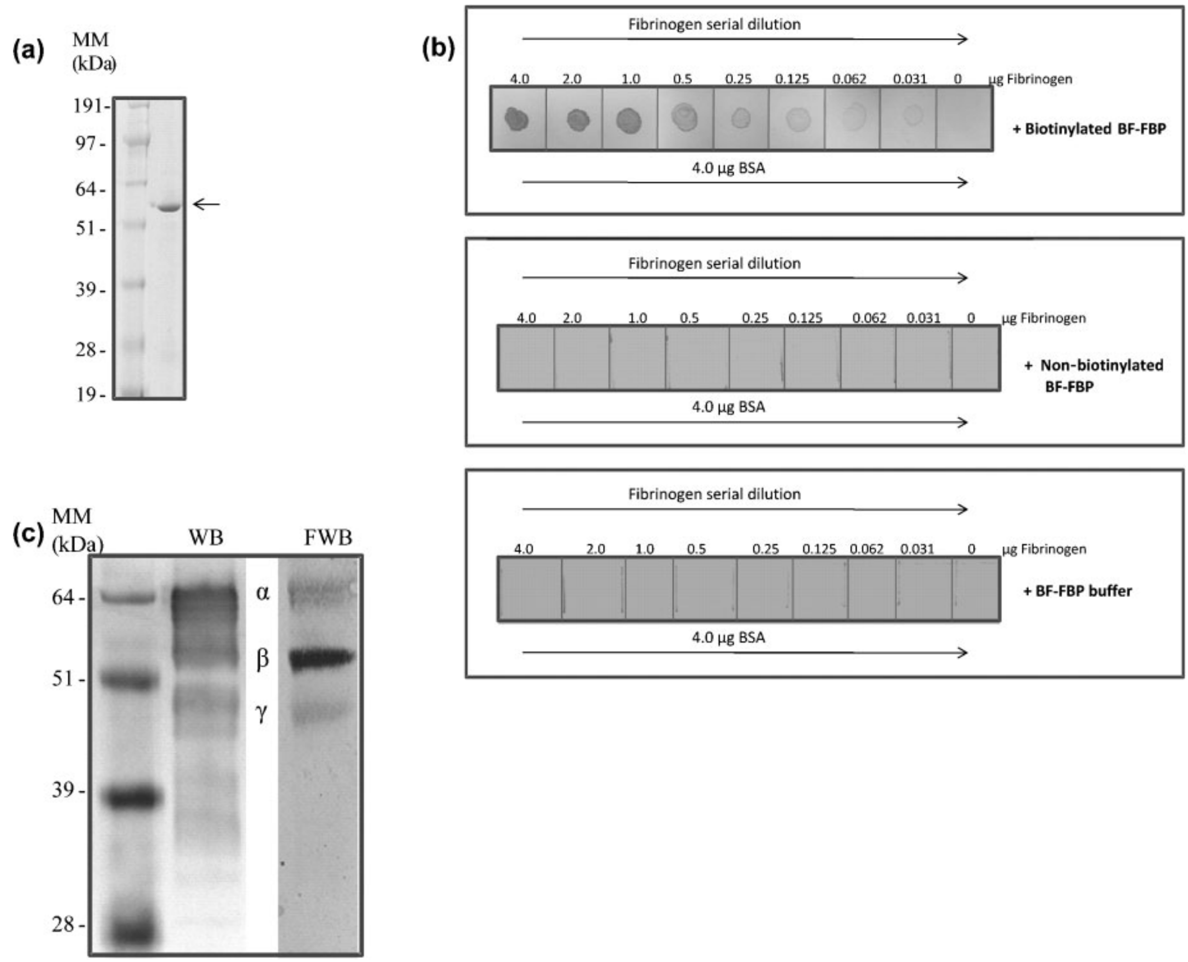

Fig. 2. Fibrinogen-binding potential of the *B. fragilis* NCTC9343 rBF-FBP. (a) GelCode Blue-stained SDS-PAGE gel of purified His-tagged rBF-FBP. (b) Dot-blot analysis of rBF-FBP, demonstrating binding to human fibrinogen. Human fibrinogen dot blot (twofold dilution; 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 μg fibrinogen), reacted with biotinylated His-tagged rBF-FB protein, native (non-biotinylated) rBF-FB protein, or protein buffer only and streptavidin–alkaline phosphatase. The results are representative of two separate experiments. (c) Immunoblots to detect binding of the biotinylated His-tagged rBF-FB protein to the Aα-, Bβ- and/or γ-chains of human fibrinogen. Blots were reacted with goat anti-human fibrinogen polyclonal antibody and rabbit anti-goat IgG (whole molecule) alkaline phosphatase conjugate (WB) and biotinylated rBF-FB protein followed by streptavidin–alkaline phosphatase (FWB).

Fibrinogenolysis activity is associated with supernatants from *B. fragilis* broth culture

Initial fibrinogen Aα-chain hydrolysis was observed with concentrated exponential phase supernatants from the enterotoxin-negative *B. fragilis* strains NCTC9343, 638R and YCH46 (Fig. 3a), confirming that all three express non-cell-associated proteases during exponential phase capable of degrading human fibrinogen. SDS-PAGE analysis of fibrinogen degradation by outer membrane protein extracts from strains NCTC9343 and YCH46 reproducibly failed to detect hydrolysis of the fibrinogen chains (data not shown); however, partial hydrolysis of the Aα-chain by the outer membrane protein extracts of strain 638R was observed following 24 h incubation (Fig. 3b). This was confirmed by measuring SDS-PAGE band intensity using ImageJ software. The relative percentage band intensity of the fibrinogen chains at t=24 h compared with t=0 h for the Aα-chain was 50.4 %, for the Bβ-chain 92.0 % and for the γ-chain 85.5 %

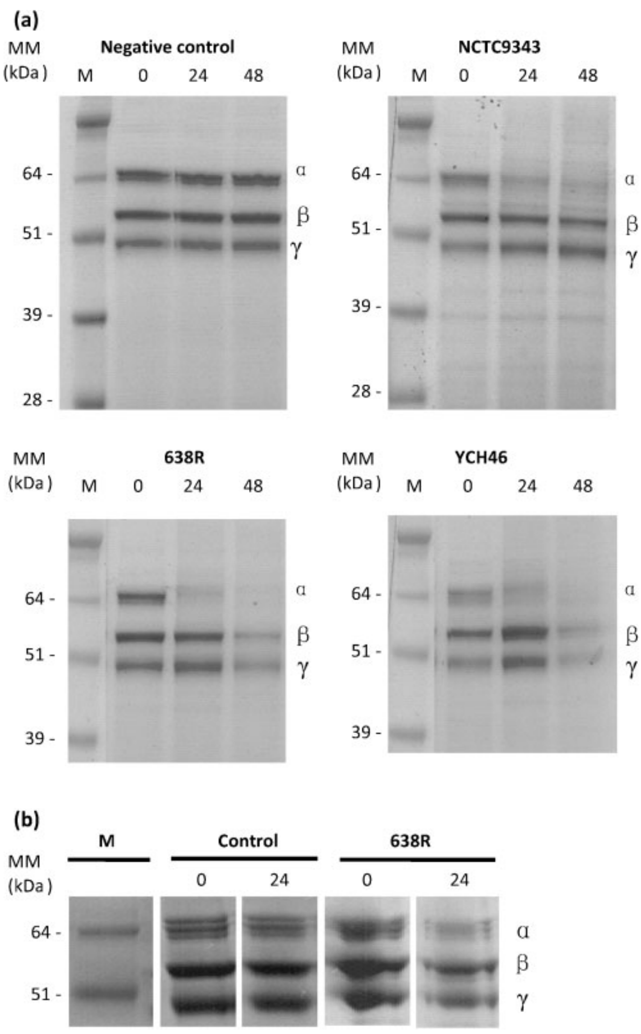

Fig. 3. Comparison of the fibrinogenolytic potential of concentrated exponential phase supernatants and outer membrane protein extracts of *B. fragilis*. (a) SDS-PAGE gel analysis of fibrinogen degradation by *B. fragilis* NCTC9343, 638R and YCH46 exponential phase supernatants over 48 h. M, molecular mass markers. (b) SDS-PAGE gel analysis of fibrinogen degradation by *B. fragilis* 638R outer membrane protein extracts over 24 h. Lanes are labelled with time points (h); M, molecular mass markers. The results are representative of two separate experiments.

Supernatants from exponential phase cultures of *B. fragilis* strains NCTC9343, 638R and YCH46 were studied further in fibrinogen zymography experiments. Fibrinogenolytic activity (at ~45 and 50 kDa) was detected in the exponential phase supernatants from BHI-S-grown cultures (Fig. 4a), and also in stationary phase supernatants (not illustrated) from strains NCTC9343 and 638R following protein refolding and activation in serine protease and metalloprotease activation buffers. Activity at the same molecular mass was also evident in DM-grown culture supernatants (not illustrated). Inhibition of this activity was observed after pre-incubation with the metalloprotease inhibitor, EDTA, but not the serine protease inhibitor PMSF (Fig. 4b). Activity was compared with a negative control sample of concentrated media broth and positive control samples of supernatants lacking inhibitor. These results suggest that the two exponential phase fibrinogenolytic proteases of NCTC9343 and 638R belong to the metalloprotease catalytic type of enzyme. In contrast, only faint fibrinogenolytic activity (~45 kDa) was detected in cysteine protease activation buffer with strainYCH46 (not illustrated).

MS analyses of proteins in the 35–60 kDa molecular mass range, obtained by excision of Gelcode blue-stained SDS-PAGE gels of 100-fold concentrated supernatants from *B. fragilis* mid-exponential phase cultures grown in defined medium, did not, however, reveal any proteases predicted by the complete genome sequence annotation. Predicted *B. fragilis* proteins that were detected included putative RagB (Hanley *et al.*, 1999) homologues encoded by BF2196, BF0595 and BF0597. These have predicted signal sequences with lipoprotein lipid attachment sites and are paired with putative *ragA* genes that contain TonB-dependent receptor motifs. In *Porphyromonas gingivalis*, RagA is an immunodominant surface antigen. BF0595 and 0597 are part of a complex invertible region (IRCC) shufflon, active in the shotgun sequence of NCTC9343 and containing four *ragA/B*-like pairs of genes each capable of shuffling from silent loci into an expression locus as a result of DNA inversion (Cerdeno-Tarraga *et al.*, 2005). BF0991 encodes a hypothetical protein located in a cluster of four genes, all with predicted signal peptides, downstream of a putative transcriptional regulator; these genes include a putative outer membrane protein and a gene with similarity to a membrane-associated haemolysin transporter, suggesting that this region is involved in export. In addition glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified. Although recognized as a classical cytoplasmic housekeeping enzyme, GAPDH has also been reported to

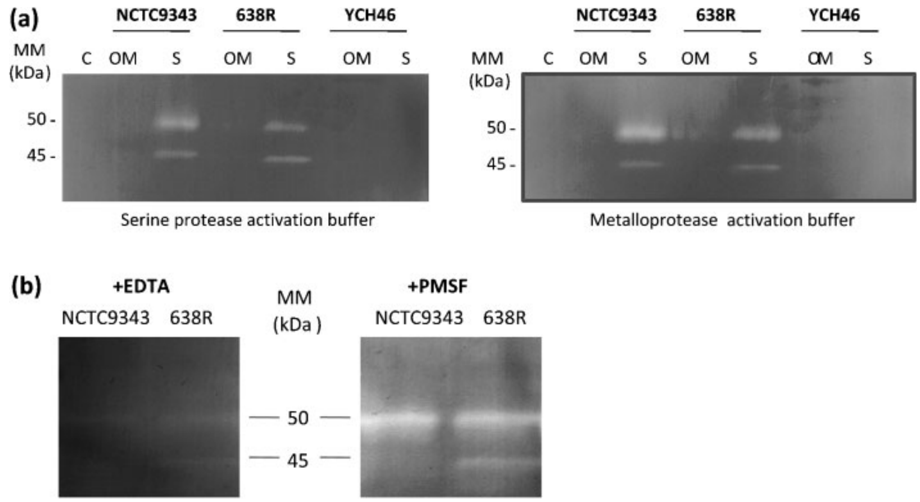

Fig. 4. Fibrinogen zymography of *B. fragilis* NCTC9343, 638R and YCH46. (a) Exponential phase supernatants (S), outer membrane extracts (OM) and concentrated BHI-S media broth alone (C) activated in serine protease and metalloprotease activation buffers. (b) Fibrinogen zymography of *B. fragilis* NCTC9343 and 638R concentrated exponential phase supernatants incubated with the protease inhibitors EDTA (10 mM) and PMSF (10 mM). The results are representative of three separate experiments.

be located on the cell surface of, and secreted by, enterohaemorrhagic and enteropathogenic *E. coli* (Egea *et al.*, 2007). The concentrated DM culture supernatant would have contained outer membrane vesicles which originate from the outer membrane by budding (Lutton *et al.*, 1991) and have demonstrable enzymic activity (Patrick *et al.*, 1996).

Comparison of the fibrinogenolytic potential of *B. fragilis* clinical isolates

To determine the relationship between fibrinogenolytic activity and growth phase, the three fully sequenced strains (NCTC9343, 638R and YCH46) were compared by the combination of TVCs, SDS-PAGE and immunoblot analysis during 48 h growth in BHI-S broth. Fibrinogen alone was stable in BHI-S during this time period, whereas partial hydrolysis of the Aα-chain was observed 6–12 h post-inoculation (late exponential phase) in the three fully sequenced strains, with complete degradation by 12–24 h (early to mid stationary phase; Fig. 5, Table 1). Initiation of Bβ-chain hydrolysis by these strains occurred 12–24 h after incubation (early to mid stationary phase) and was complete by 33–48 h (late stationary/early death phases; Fig. 5). Degradation of the fibrinogen γ-chain by strains NCTC9343 and 638R was not as obvious after SDS-PAGE analysis of the supernatants. The immunoblots, however, indicated that a noticeable proportion of the γ-chain was hydrolysed by 33 h, and epitopes detected by the anti-human fibrinogen antiserum were completely degraded by 48 h with NCTC9343 and 638R. TVCs indicated that γ-chain degradation by both these strains commenced during late stationary phase. In contrast, with strain YCH46 (Fig. 5c), hydrolysis was observed by 12–24 h post-inoculation, during early to mid stationary phase. This suggests that YCH46 expresses proteases additional to or different from those of the other two strains. Fibrinogen degradation profiles for a further four abscess and two bacteraemia *B. fragilis* clinical isolates (Table 1) revealed initial degradation of the Aα-chain commencing during the late exponential growth phase, followed by the stationary phase cleavage of the Bβ-chain and partial, often less intense, degradation of the γ-chain. A different pattern was observed for bacteraemia isolate SP2, which degraded the Bβ- and γ-chains prior to the Aα-chain degradation, and initiated degradation of all three chains within 6–12 h, corresponding to the mid–late exponential growth phase (Fig. 6).

DISCUSSION

This is the first study, to our knowledge, to demonstrate the binding of human fibrinogen to the surface of *B. fragilis* and the identification and purification of BF-FB, a putative surface lipoprotein encoded by BF1705 of *B. fragilis* strain NCTC9343 (Cerdeno-Tarraga *et al.*, 2005), and also identified in the genomes of strains 638R (S. Patrick and others, unpublished results) and YCH46 (Kuwahara *et al.*, 2004). Fibrinogen adhesion to the *B. fragilis* cell surface was clearly evident within two hours of exposure, a property that could be advantageous during infection. This rapid binding would potentially interfere with fibrinogen functions, such as abscess formation, and as a result of bacterial aggregation/clump formation may also confer resistance to phagocytosis (Kapral, 1966; Dominiecki &

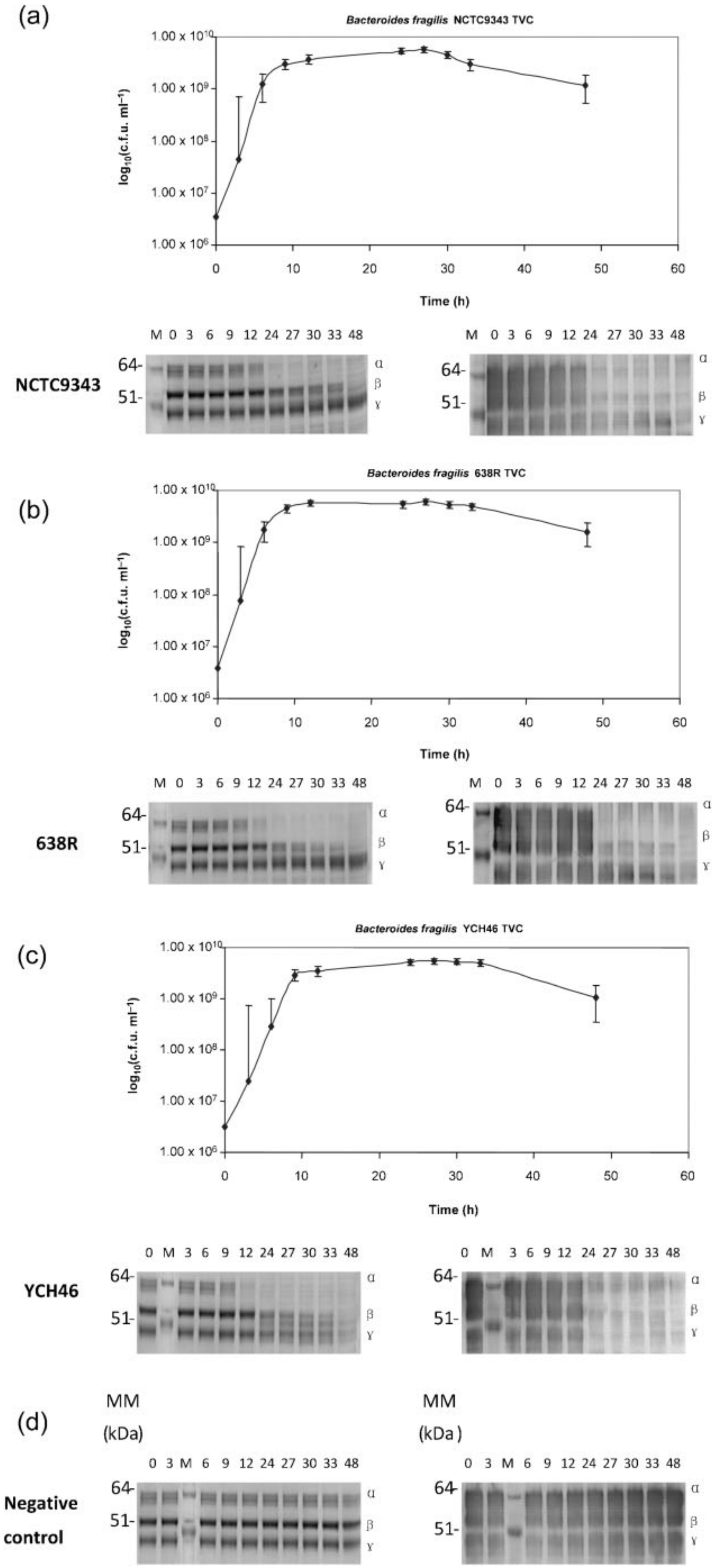

Fig. 5. Comparison of the fibrinogenolytic potential of *B. fragilis* cultures. SDS-PAGE gel analysis (left-hand panels) and immunoblot analysis (right-hand panels) of fibrinogen degradation by *B. fragilis* NCTC9343 (a), 638R (b) and YCH46 (c) for 0–48 h and corresponding growth curves at each time point during 48 h fibrinogen degradation experiments (mean ± SE of six replicate experiments). Immunoblots were reacted with goat anti-human fibrinogen polyclonal antiserum. Lanes are labelled with time points (h); M, molecular mass markers. (d) Negative control. Note that fibrinogen was stable over 48 h (negative control), whereas fibrinogen incubated with the three *B. fragilis* strains was degraded.

Table 1. Time-course of the initiation of fibrinogen chain degradation by nine *B. fragilis* strains as determined by a combination of SDS-PAGE, immunoblotting and TVC analyses

Isolate	Time period to initiation of fibrinogen degradation (h)		
	Aα-chain	Bβ-chain	γ-Chain
SP2*	6–12	0–6‡	0–6‡
YCH46*, 638R†	6–12	12–24	12–24
LS98*, DK9†	6–12	12–24	24–48
NCTC9343†	6–12	12–24	30–48
LS27†	12–24	12–24	12–24
SP1*	6–12	6–12	Stable
LS66†	6–12	12–24	Stable

*Blood culture isolate.
†Abscess isolate.
‡Note that degradation of fibrinogen Bβ- and γ-chains was initiated before that of the Aα-chain.

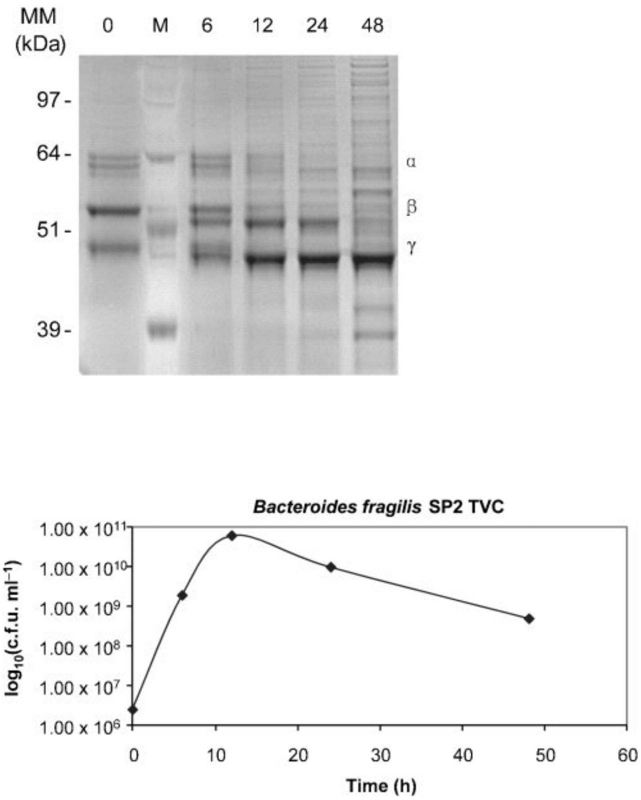

Fig. 6. Coomassie blue SDS-PAGE gel analysis of fibrinogen degradation by *B. fragilis* SP2 over 48 h (top panel) with the corresponding growth curve at each time point (lower panel). Note the fibrinogen degradation 0–6 h following exposure of *B. fragilis* to fibrinogen and Bβ- and γ-chain degradation prior to Aα-chain degradation. The results are representative of a minimum of two replicate experiments.

Weiss, 1999). The genetic conservation of BF-FB suggests that it is also involved in survival within the gastrointestinal tract. Purified recombinant BF-FB binds to human fibrinogen through interaction predominantly with the Bβ-chain. The 95 kDa cell-surface fibrinogen-binding protein orthologue BspA of *T. forsythia* contains 14 LRR motifs (Sharma *et al.*, 1998), in comparison with BF-FB, which has only two LRRs. Whether the LRRs are involved in multimer formation or are directly involved in fibrinogen binding is unknown, but the reduction in LRR motifs in *B. fragilis* clearly does not preclude fibrinogen binding by the recombinant protein.

NCTC9343, 638R and YCH46 all exhibited non-cell-associated fibrinogenolytic activity in concentrated supernatants. As these strains are enterotoxin-negative, this activity was not associated with enterotoxin. Zymography revealed metalloprotease activity associated with non-cell-associated proteins of 45 and 50 kDa in NCTC9343 and 638R, and weaker activity in cysteine activation buffer with strain YCH46. As YCH46 in culture degraded fibrinogen as effectively as NCTC9343 and 638R, clearly *B. fragilis* has more than one potential mechanism for fibrinogen degradation. Chen *et al.* (1995) have reported that a cellular extract from *B. fragilis* YCH46 degrades fibrinogen when examined by SDS-PAGE analysis. Although we also observed degradation by this strain, we did not identify the 100 kDa serine-thiol-like protease of *B. fragilis* YCHC46 reported by Chen and colleagues in our supernatants or our zymography studies; nor was a putative corresponding cell-surface or secreted 100 kDa serine-thiol-like protease candidate gene identified by *in silico* genome analyses. It is possible that the serine-thiol-like protease purified by Chen *et al.* (1995) was intracellular in origin, as only whole-cell extracts were examined. Examination of these strains and a further six clinical isolates by culture in fibrinogen-containing medium showed that, with one exception, initiation of degradation of the fibrinogen Aα-chain occurred first during the exponential growth phase, followed by Bβ-chain hydrolysis. Proteolysis was therefore occurring during active growth of the bacteria and was not simply an activity resulting from bacterial cell death and the release of cytoplasmic components. Degradation of the γ-chain was more variable. It remained stable with some isolates and was not degraded until stationary phase with others. Interestingly, blood culture isolate SP2 rapidly degraded fibrinogen Bβ- and γ-chains. Whether this is related to an enhanced capability for dissemination and bacteraemia remains to be determined, as there was no common pattern for all bacteraemia isolates. These data demonstrate that different isolates may produce more than one or different types of fibrinogen-degrading proteases.

Fibrinogen molecules are composed of six polypeptide chains, two of each of the Aα-, Bβ- and γ-chains, linked by disulphide bonds. One of each of the Aα, Bβ and γ chains extends outwards to either side of the central E domain (which contains the N termini), forming two globular outer D domains. The C termini of the Bβ- and γ-chains are located within the globular D domains. In contrast, the Aα-chains extend outside the globular D domains, back inwards towards the N termini in the central E domain (Doolittle, 1984; Weisel *et al.*, 1985; Weisel, 2005). The difference in the Aα-chain location may therefore account for its increased predisposition to proteolytic attack by certain proteases. Interestingly, *Pseudomonas aeruginosa* elastase (Komori *et al.*, 2001) and *Streptococcus pyogenes* extracellular cysteine protease (Matsuka *et al.*, 1999) both target the Aα-chain. A reduction or absence of this subunit alone, even in the presence of normal Bβ and γ fibrinogen, is sufficient to adversely affect clot formation in the condition known as afibrinogenaemia (Neerman-Arbez, 2001). Thus, prevention of clot formation may be a crucial mechanism by which *B. fragilis* evades the host abscess-forming defence mechanism.

MS analysis of proteins in the molecular mass region corresponding to the detected fibrinogenolytic activity did not reveal a candidate enzyme containing a signal sequence. GAPDH was, however, detected. Classical cytoplasmic housekeeping enzymes such as glycolytic enzymes, including enolase and GAPDH, have been shown to be localized to the outer surface of some pathogenic microbes and have been related to virulence (Pancholi & Chhatwal, 2003). The presence of putative lipoproteins, such as RagB homologues, suggests that these are likely to be associated with outer membrane vesicles, which would have been present in the broth culture supernatants.

The expression of the fibrinogen-binding protein (BF-FBP) and fibrinogenolytic proteases by *B. fragilis*, described herein, may represent important virulence factors in *B. fragilis*, allowing the bacteria to slow down or prevent abscess formation and promote abscess dissolution, resulting in dissemination of infection, and may potentiate the rapid release of large numbers of *B. fragilis* into the host circulation, resulting in bacteraemia.

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